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Trimethylation and Acetylation of -Catenin at Lysine 49 Represent Key Elements in ESC Pluripotency

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Abstract: Wnt/ -catenin signaling is required for embryonic stem cell (ESC) pluripotency by inducing mesodermal differentiation and inhibiting neuronal differentiation; however, how -catenin counterregulates these differentiation pathways is unknown. Here, we show that lysine 49 (K49) of -catenin is trimethylated (-catMe3) by Ezh2 or acetylated (-catAc) by Cbp. Significantly, -catMe3 acts as a transcriptional co-repressor of the neuronal differentiation genes sox1 and sox3, whereas -catAc acts as a transcriptional co-activator of the key mesodermal differentiation gene t-brachyury (t-bra). Furthermore, -catMe3 and -catAc are alternatively enriched on repressed or activated genes, respectively, during ESC and adult stem cell differentiation into neuronal or mesodermal progenitor cell lineages. Importantly, expression of a -catenin K49A mutant results in major defects in ESC differentiation. We conclude that -catenin K49 trimethylation and acetylation are key elements in regulating ESC pluripotency and differentiation potential.

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Report

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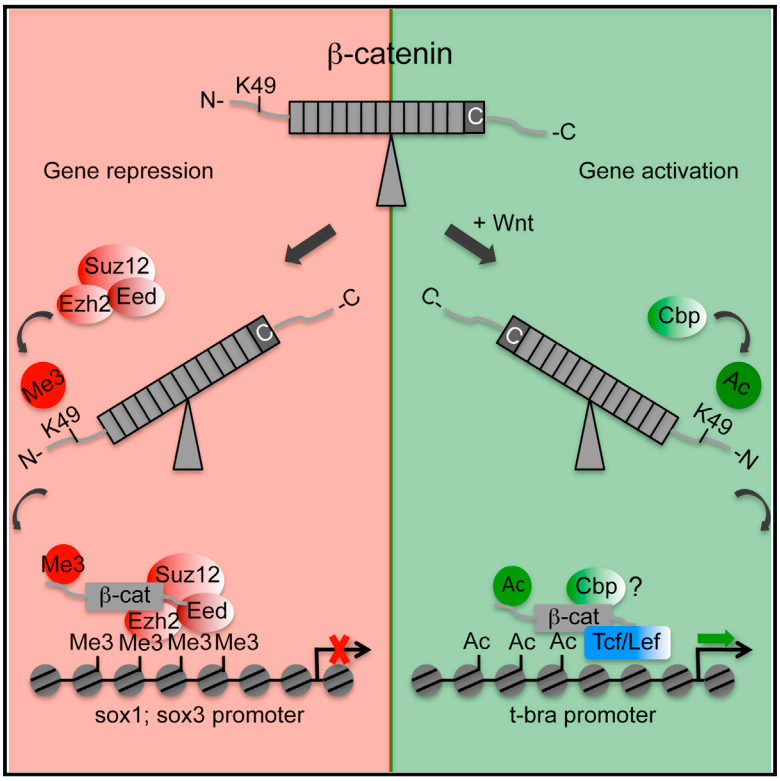
Graphical Abstract



Highlights

- Wnt/β-catenin signaling is required for ESC pluripotency and differentiation
- Lysine 49 of β-catenin is trimethylated by Ezh2 or acetylated by Cbp
- These modifications are alternatively enriched at repressed or activated genes
- Mutation of lysine 49 impairs ESC pluripotency and differentiation

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In Brief

In ESCs, Wnt/β-catenin signaling induces mesodermal and inhibits neuronal differentiation. Hoffmeyer et al. show that β-catenin is trimethylated by Ezh2 at K49 and associated with gene repression. K49 is also acetylated by Cbp, resulting in the activation of genes. Thus, post-translational modifications of K49 represent a molecular switch for β-catenin function.

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Trimethylation and Acetylation of β-Catenin at Lysine 49 Represent Key Elements in ESC Pluripotency

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SUMMARY

Wnt/β-catenin signaling is required for embryonic stem cell (ESC) pluripotency by inducing mesodermal differentiation and inhibiting neuronal differentiation; however, how β-catenin counter-regulates these differentiation pathways is unknown. Here, we show that lysine 49 (K49) of β-catenin is trimethylated (β-catMe3) by Ezh2 or acetylated (β-catAc) by Cbp. Significantly, β-catMe3 acts as a transcriptional co-repressor of the neuronal differentiation genes *sox1* and *sox3*, whereas β-catAc acts as a transcriptional co-activator of the key mesodermal differentiation gene *t-brachyury* (*t-bra*). Furthermore, β-catMe3 and β-catAc are alternatively enriched on repressed or activated genes, respectively, during ESC and adult stem cell differentiation into neuronal or mesodermal progenitor cell lineages. Importantly, expression of a β-catenin K49A mutant results in major defects in ESC differentiation. We conclude that β-catenin K49 trimethylation and acetylation are key elements in regulating ESC pluripotency and differentiation potential.

INTRODUCTION

Historically, β-catenin was first identified in association with the cell adhesion molecule E-cadherin (Vestweber and Kemler, 1984). In the cadherin-catenin adhesion complex, β-catenin binds directly to the cytoplasmic domain of E-cadherin and mediates via α-catenin the anchorage to the actin-based cytoskeleton (Ozawa et al., 1989). During recent years, a second role of β-catenin in the Wnt signaling pathway became increasingly apparent (Nelson and Nusse, 2004).

The canonical Wnt signaling pathway plays a major role in embryonic stem cell (ESC) and adult stem cell differentiation, and dysregulation of this pathway is frequently observed in human cancers (Reya and Clevers, 2005). β-Catenin is a central component of the Wnt pathway, acting as a transcriptional co-activator with LEF/TCF transcription factors to control target gene expression. Wnt/β-catenin signaling is required for pluripotency in order to maintain ESCs in the undifferentiated state (ten Berge et al., 2011; Ying et al., 2008), the induction of mesodermal differentiation (Gadue et al., 2006), and, at the same time, inhibition of neuronal differentiation (Haegele et al., 2003; Kielman et al., 2002; Verani et al., 2007). These findings suggest a complex role for β-catenin function in directing opposing cell fates in ESCs.

It is well known that gene expression is controlled by changes in post-translational modifications of histones, such as methylation by the polycomb repressive complex 2 (PRC2). Methyltransferases (Ezh1 and Ezh2) in the PRC2 complex modify lysine 27 of histone 3 (H3K27Me3) (Morey and Helin, 2010), and H3K27Me3 represses developmentally regulated genes (O'Carroll et al., 2001; Sparmann and van Lohuizen, 2006) and is required for stem cell differentiation (Pasini et al., 2007). Ezh2 also mediates methylation of non-histone proteins in a PRC2-independent manner (He et al., 2012; Kim et al., 2013), and an interaction of Ezh2 with the canonical Wnt signaling pathway has been reported (Jung et al., 2013).

Here, we show that β-catenin is in complex with PRC2 components in ESCs and that it is trimethylated by Ezh2 at K49. Importantly, we show that β-catMe3 represses neuronal differentiation genes *sox1* and *sox3*. β-Catenin K49 has also been reported to be acetylated by CREB-binding protein (Cbp) (Wolf et al., 2002), and we show the acetylation of K49 by Cbp is involved in the activation of the mesodermal differentiation gene *t-bra*. Taken together, our data indicate that post-translational modification of K49 of β-catenin functions as a molecular switch for β-catenin function, determining whether it acts as a co-activator or a co-repressor. We hypothesize that this pathway for regulating β-catenin function is important for maintaining ESC pluripotency and differentiation potential.

RESULTS

Ezh2 Methylates β-Catenin at Lysine 49

Using a SILAC-based approach to search for nuclear interaction partners of β-catenin, we identified several components of

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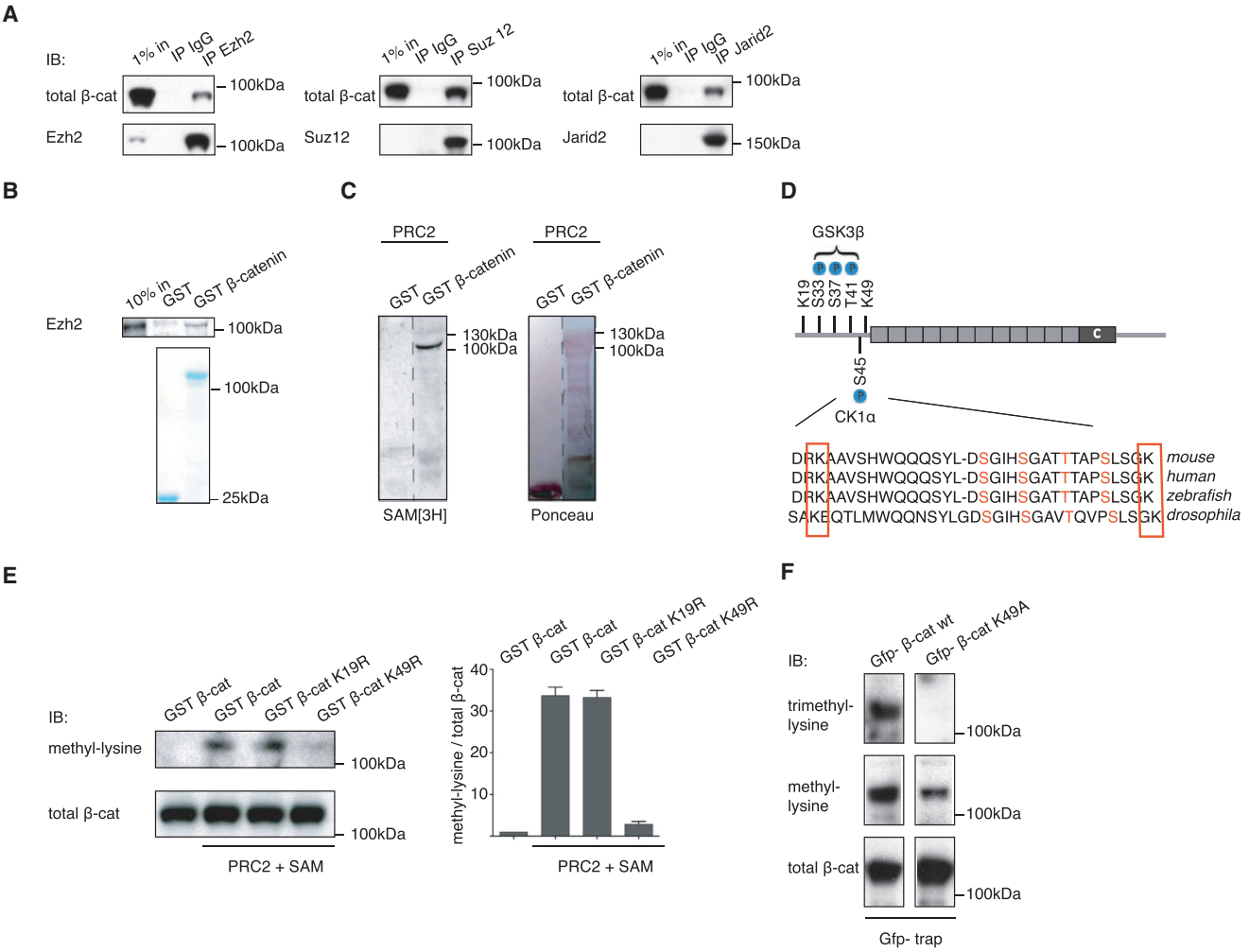


Figure 1. Ezh2 Methylates β-Catenin at Lysine 49

(A) β-catenin co-purifies with components of the PRC2 complex Ezh2, Suz12, and Jarid2 from nuclear extracts of ESCs.

(B) In vitro-translated Ezh2 associates with glutathione S-transferase (GST)-β-catenin.

(C) Autoradiography showing in vitro methylation of GST-β-catenin incubated with active PRC2 complex and S-adenosyl-L-methionine, S-[methyl-^{3}H] (SAM[^{3}H]). Ponceau stain shows input.

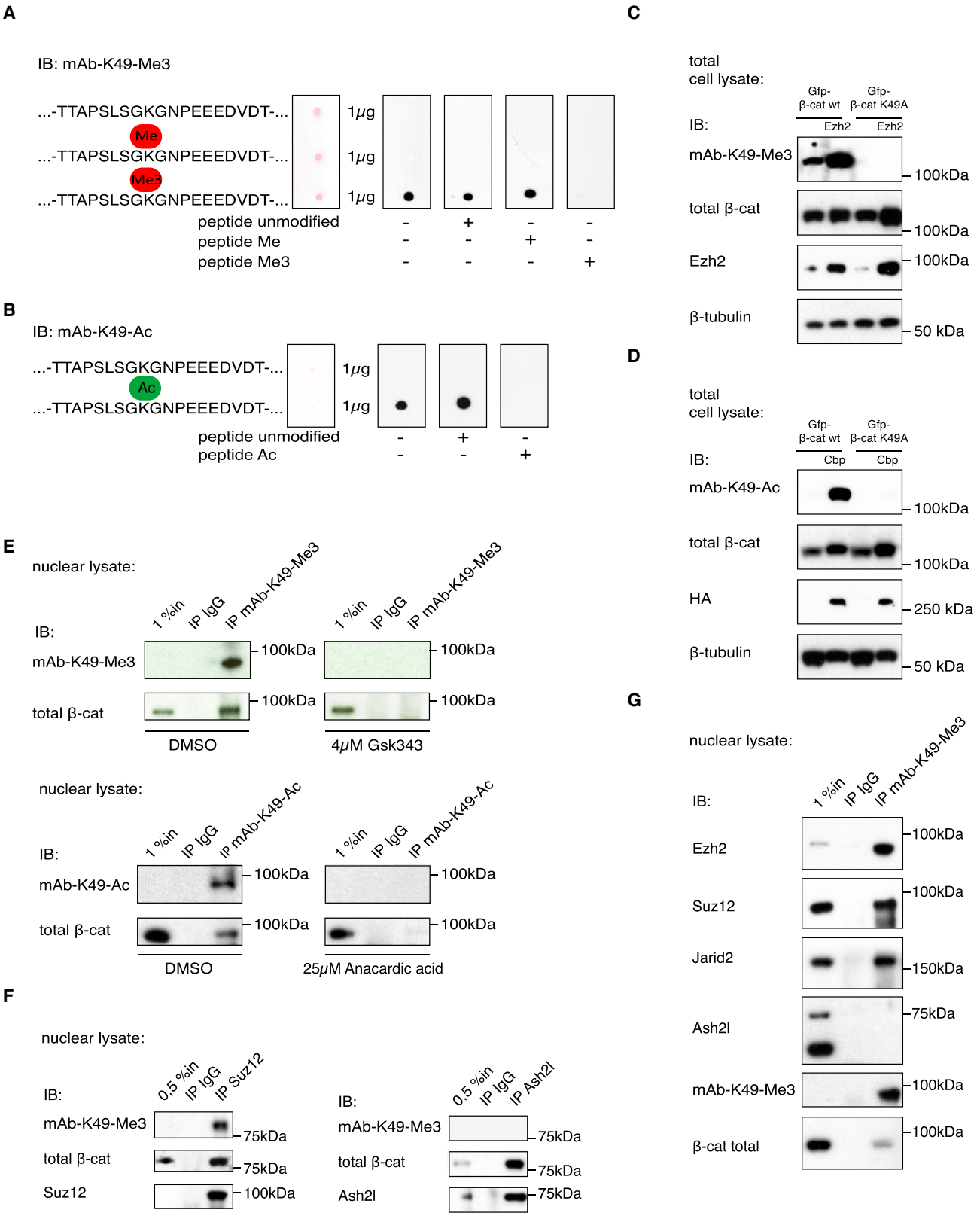
(D) Species alignment of amino acids encoded by exon 3 of β-catenin; the position of K19 and K49 is conserved among species.

(E) In vitro methylation of GST-β-catenin or GST-β-catenin carrying lysine (K)-to-arginine (R) mutations at K19 or K49 with active PRC2 complex and SAM. Left: detection of methylated lysines and total β-catenin by immunoblot. Right: quantification of two independent experiments using ImageJ software.

(F) GFP-β-catenin wild-type (Gfp-β-cat WT) or K49A mutation transfected into Hek 293 cells. Gfp-Trap followed by detection of trimethylated or methylated lysines and β-catenin by immunoblot.

the PRC2 complex (Hoffmeyer, 2012). This interaction was verified by the co-immunoprecipitation of β-catenin with Suz12 and Ezh2, but not Jarid2, from nuclear extracts of ESCs (Figure 1A). Recombinant β-catenin also pulled-down Ezh2 (Figure 1B) and Suz12 (not shown), but not Jarid2 or Eed (Figure S1A), from ESC lysates. Significantly, β-catenin was identified as a substrate of Ezh2 using an in vitro methylation assay with PRC2 and recombinant β-catenin (Figure 1C), and β-catenin deletions indicated that the methylation site was within exon 3 of the β-catenin gene (Figure 1D). Within exon 3, K19 and K49 are possible targets for methylation, and we identified K49, not K19, as the site using a K− > R mutation that blocked β-catenin methylation by PRC2 (Figure 1E). Significantly, a β-cat-K49A mutant was mono- and dimethylated but not trimethylated (Figure 1F). Attempts to demonstrate methylation by mass spectrometry were not successful, because trypsin cannot digest K49 of β-catenin when it is methylated.

β-Catenin K49 is close to well-characterized phosphorylation sites for CK1α (T45) and GSK3β (S33, S37, T41), indicating a possible molecular cross-talk between phosphorylation and methylation. Furthermore, methylation of β-catenin by Ezh2 at K49 may activate Wnt/β-catenin signaling (Zhu et al., 2016). However, and ignored in this previous report, K49 is acetylated



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by Cbp (Wolf et al., 2002). Thus, post-translational modification of K49 by either acetylation or methylation may be biologically relevant to the function of β-catenin.

Monoclonal Antibodies Specific for Methylated and Acetylated Lysine 49 Reveal Complex Specificity for β-catMe3

To gain further insight into the role of these β-catenin modifications, we raised rat monoclonal antibodies specific for β-catenin K49 methylation (mAb-K49-Me3) (Figure 2A) and acetylation (mAb-K49-Ac) (Figure 2B). A monoclonal antibody that recognized unmodified, acetylated, mono- and trimethylated β-catenin (mAb-β-cat-total) was used as a control (Figure S1D).

The specificity of mAb-K49-Me3 and mAb-K49-Ac was tested. First, Hek293 cells transiently expressing Gfp-tagged wild-type β-catenin (Gfp-β-cat WT) or β-catenin in which K49 was substituted by alanine (Gfp-β-cat K49A) were co-transfected with either Ezh2 or Cbp. Western blotting of cell lysates showed that mAb-K49-Me3 and mAb-K49-Ac detected β-catMe3 and β-catAc, respectively, but not Gfp-β-cat K49A (Figures 2C and D). Co-expression of Ezh2 enhanced the level of β-catMe3 (Figure 2C), and β-catAc was detected upon co-expression of Cbp (Figure 2D). Second, mAb-K49-Me3 and mAb-K49-Ac immunoprecipitated β-catMe3 and β-catAc, respectively, from nuclear extracts of mouse ESCs (Figure 2E). Third, mAb-K49-Me3 and mAb-K49-Ac detected β-catMe3 and β-catAc in immunoblots of whole cell lysates from Hek293 cells and the human carcinoma cell line, SW480 (Figure S1C). Fourth, mAb-K49-Me3 and mAb-K49-Ac did not immunoprecipitate β-catMe3 and β-catAc following inhibition of Ezh2 or Cbp enzymatic activity with the specific inhibitors Gsk343 and anacardic acid, respectively (Figures 2E and S1B). However, mAb-K49-Me3 detected β-catMe3, albeit at a greatly reduced level, in ESCs deficient for Ezh2 ($Ezh2^{-/-}$) (Figure S2A), indicating that other HKMTs may contribute to β-catenin trimethylation. Indeed, Ezh1 can trimethylate β-catenin (Figure S2C), and a double null for Ezh1 and Ezh2 resulted in no detectable β-catMe3 (Figures S2B and S2D).

We had shown previously that β-catenin associated with Ash1, a component of the MLL complex (Hoffmeyer et al., 2012). However, Ash21 and β-catMe3 did not co-immunoprecipitate (Figure 2F). In contrast, β-catMe3 co-immunoprecipitated with the PRC2 complex components Ezh2, Suz12, and Jarid2 (Figure 2G). Together, these results indicate strongly that Ezh2 trimethylates β-catenin at K49 in cells, which is detected with mAb-K49-Me3.

Post-translationally Modified β-Catenin Is Differentially Enriched at Promoter Regions of Key Developmental Genes

β-Catenin is the main transcriptional co-activator in the Wnt/β-catenin signaling pathway. Because both β-catMe3 and β-catAc were detected in the nuclei of ESCs (Figures 2E and 2F), we investigated the role of these post-translational modifications of β-catenin K49 in the transcriptional regulation of β-catenin target genes in mouse ESCs.

WT and β-catenin-deficient ($\beta\text{-}Cat^{-/-}$) ESCs were treated with either Wnt3a or the Wnt-mimicking reagent CHIR99021. After 48 hr, cells were collected and mRNA levels of the mesodermal marker gene t-brachyury (t-bra), and the neuronal marker genes sox1 and sox3 were measured. The mRNA levels of the t-bra were markedly increased after Wnt3a or CHIR99021 treatment, whereas the mRNA levels of sox1 and sox3 were strongly reduced (Figures 3A and S3A, left panels). These changes in mRNA expression were dependent on β-catenin expression (Figures 3A and S3A, right panels).

We tested whether these changes in t-bra, sox1, and sox3 mRNA levels were due to a direct interaction with either β-catAc or β-catMe3. Chromatin immunoprecipitation (ChIP) experiments with mAb-K49-Me3, mAb-K49-Ac, and mAb-β-cat-total revealed a direct association of β-catMe3 with all three promoters in untreated WT ESCs (Figure 3B). Importantly, after CHIR99021 treatment, β-catMe3 was depleted from the t-bra promoter region but remained on the sox1 and sox3 promoters (Figure 3C). As a control for the mAb-K49-Me3 antibody, ChIP was performed in GSK343-treated WT ES and $\beta\text{-}Cat^{-/-}$ and $Ezh2^{-/-}$ ESCs (Figure 3C). ChIP of Ezh2, Suz12, and Eed revealed that PRC2 core components and β-catMe3 were bound to the same promoter regions in a CHIR99021-dependent manner (Figures S3D–S3F) with H3K27Me3 (Figure S3B).

After induction of the Wnt pathway with CHIR99021, β-catAc was selectively detected at the t-bra promoter region, but not at the sox1 or sox3 promoter regions (Figure 3B), with H3K27Ac (Figure S3C). ChIP with mAb-β-cat-total to detect unmodified, acetylated, and methylated β-catenin revealed that β-catenin was present on all three promoters, and there was no change in levels after CHIR99021 treatment of WT ESCs. β-Catenin was also detected in $Ezh2^{-/-}$ ESCs. $\beta\text{-}Cat^{-/-}$ ESCs were used as a negative control (Figure 3D).

In summary, CHIR99021-dependent induction of t-bra (Figures 3A and S3A) correlated with the enrichment of β-catAc and H3K27Ac (Figures 3B and S3C), but the depletion of trimethylated H3K27, β-catMe3, and PRC2 complex components (Figures 3C, S3B, and S3D–S3F) at the t-bra promoter region.

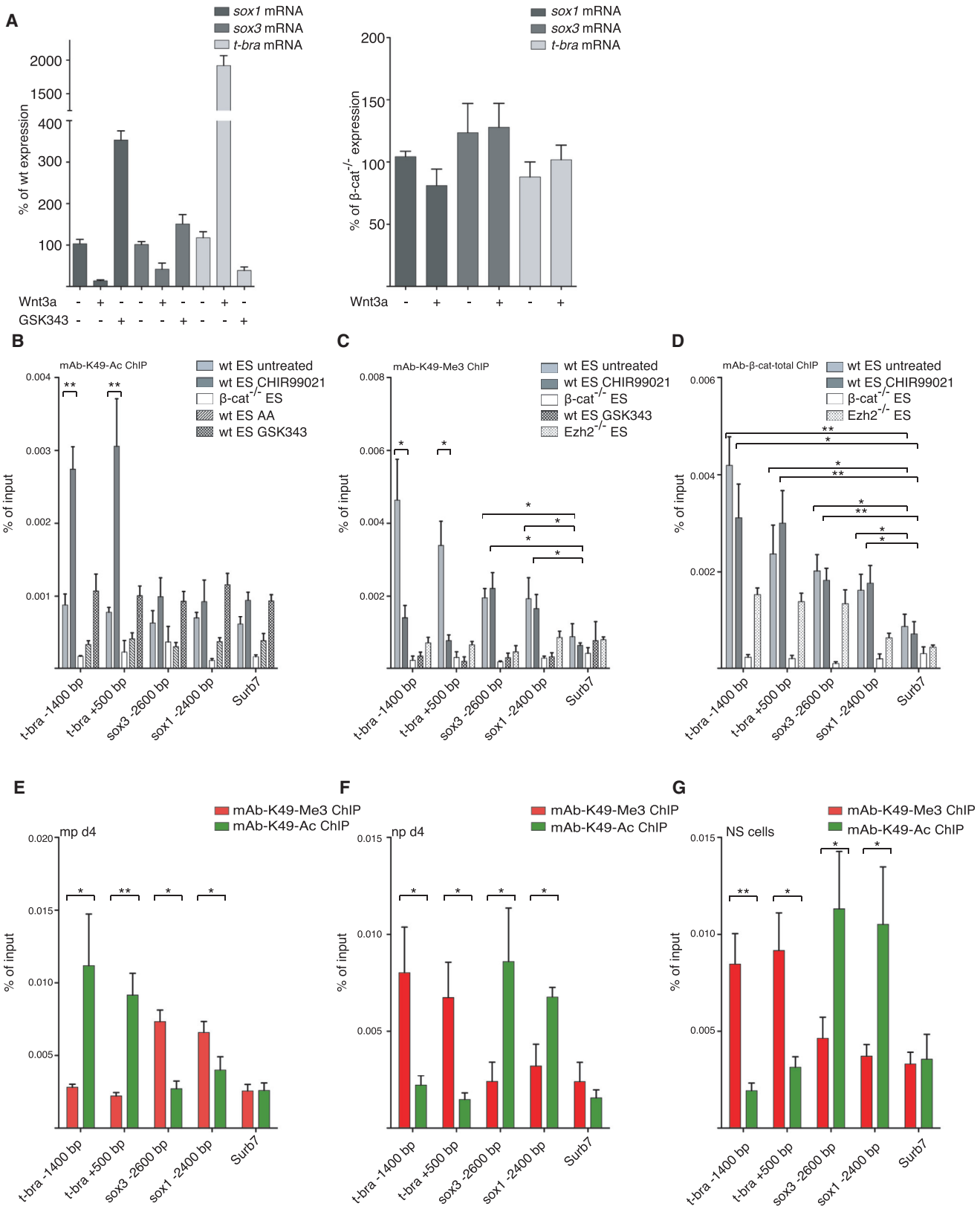
Figure 2. Monoclonal Antibodies Specific for Methylated and Acetylated Lysine 49 Reveal the Specificity of PRC2 Complex for β-catMe3

(A and B) Characterization of rat mAbs against methylated (mAb-K49-Me3) and acetylated (mAb-K49-Ac) β-catenin. Dot blots with peptide competition assays for antibody specificity; mAb-K49-Me3 (A) and mAb-K49-Ac (B).

(C and D) Gfp-β-cat WT or Gfp-β-cat K49A co-transfected with Ezh2 (C) or Cbp (D) in Hek 293 cells. Immunoblot of 20 μg total protein lysate probed with mAb-K49-Me3 and Ezh2 (C) or mAb-K49-Ac and HA (D), β-catenin and β-tubulin (C and D).

(E) Immunoprecipitations with mAb-K49-Me3 (top) and mAb-K49-Ac (bottom) from nuclear extracts of ESCs. Inhibition of Ezh2 by treatment with Gsk343 followed by immunoblot with mAb-K49-Me3 and β-catenin (bottom). Inhibition of Cbp by treatment with anacardic acid followed by immunoblot with mAb-K49-Ac and β-catenin (bottom).

(F and G) Co-immunoprecipitations with Suz12 (F, left), Ash2l (F, right), and mAb-K49-Me3 (G). Immunoblot with mAb-K49-Me3, β-catenin, Suz12, and Ash2l (F and G); Ezh2 and Jarid2 (G). β-catMe3 is only detectable in complex with PRC2 (F and G).



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In contrast, the CHIR99021-dependent decrease in sox1 and sox3 mRNA levels (Figures 3A and S3A) correlated with the enrichment of β-catMe3, Ezh2, Suz12, Eed, and H3K27Me3 at the sox1 and sox3 promoter regions (Figures 3C, S3B, and S3D–S3F).

Localization of β-catMe3 and β-catAc on Target Genes during ESC Differentiation

ESCs were differentiated into neuronal or mesodermal progenitor cells (Figures S4A and S4B), and the enrichment of β-catMe3 and β-catAc at the t-bra, sox1, and sox3 promoter regions was analyzed by ChIP. In mesodermal progenitor cells, high levels of t-bra mRNA (Figure S4A) correlated with the enrichment of β-catAc (Figure 3E) and H3k27Ac (Figure S4E) at the t-bra promoter region, whereas β-catMe3 (Figure 3E) and H3K27Me3 (Figure S4F) were enriched on the promoters of the neuronal marker genes sox1 and sox3. In contrast, in the neuronal progenitor cells high levels of sox1 and sox3 mRNAs (Figure S4B) correlated with the enrichment of β-catAc (Figure 3F) and H3k27Ac (Figure S4E) at the promoter regions of the sox1 and sox3 genes, whereas β-catMe3 (Figure 3F) and H3K27Me3 (Figure S4F) were detected at the t-bra promoter. ChIP experiments with mAb-β-cat-total detected equal levels of β-catenin at the three promoter regions in both mesodermal and neuronal progenitor cells (Figure S4D). These results provide compelling evidence that β-catenin post-translationally modified at K49 is bound to the promoters of key genes involved in ESC development, and there is a change from K49 trimethylation in mesodermal progenitor cells to acetylation in neuronal progenitor cells.

We extended these studies to primary neurosphere cultures isolated from newborn mice (Figure S4C). ChIP analysis with mAb-K49-Me3, mAb-K49-Ac, and mAb-β-cat-total revealed a binding pattern of the post-translationally modified forms of β-catenin to the t-bra, sox1, and sox3 promoters that were similar to those in differentiated ESCs (Figures 3G and S4G). The enrichment of H3K27Ac and H3K27Me3 correlated with the enrichment of mAb-K49-Me3, mAb-K49-Ac, respectively (Figures S4H and S4I). Taken together, these results show that post-translationally modified forms of β-catenin K49 correlated with mesodermal and neuronal differentiation of ESCs and adult stem cells.

Lysine 49 Mutations Affect ESC Pluripotency and Differentiation

Next, we analyzed the biological relevance of post-translational modification of β-catenin K49 using the β-catenin K49A loss-of-function mutant in ESCs. Gfp-β-catenin fusion proteins (Gfp-β-cat WT or Gfp-β-catK49A) were expressed in β-cat$^{flox/-}$ ESCs and the endogenous β-catenin allele was simultaneously deleted (scheme shown in Figure S5A). Gfp-positive (Gfp-β-cat WT or Gfp-β-cat K49A) clones were analyzed by fluorescence-activated cell sorting (FACS), and clones that expressed levels of β-catenin comparable to WT ESCs were selected (Figure S5C). All β-Gfp-β-cat WT clones exhibited typical ESC morphology, although Gfp-β-cat K49A clones grew more rapidly and in a more dispersed manner than Gfp-β-cat WT clones (Figure S5B).

Analysis of t-bra, sox1, and sox3 mRNA levels in Gfp-β-cat K49A clones revealed a decrease in t-bra mRNA levels and an increase in sox1 and sox3 mRNA levels (Figure 4A). Thus, substitution of K49 by alanine was sufficient to alter the expression of the key developmental differentiation genes t-bra, sox1, and sox3. Based on this finding, Gfp-β-cat K49A cells were subjected to in vitro differentiation for 4 days. Differentiation to mesodermal progenitor cells was examined by comparing levels of t-bra, tbx6, and msgn1 mRNAs. As expected, Gfp-β-cat WT cells exhibited strong induction of t-bra, tbx6, and msgn1 mRNAs at day 4, whereas Gfp-β-catK49A failed to induce these mesodermal markers (Figure S5E). Differentiation into neuronal progenitor cells was examined by comparing levels of sox1 and sox3 mRNAs. Gfp-β-cat WT cells induced these neural markers, whereas Gfp-β-catK49A cells exhibited high levels of sox1 and sox3 expression at day 0 (d0) (Figure 4A) that did not increase further during neuronal progenitor differentiation (Figure S5D).

To obtain a more global view of changes in gene expression in Gfp-β-cat K49A clones, we used transcriptome expression profiling. We compared two ESC clones of Gfp-β-cat WT or Gfp-β-cat K49A that were either undifferentiated or differentiated into mesodermal or neuronal progenitor cells (Figures 4B and 4C). Undifferentiated Gfp-β-cat K49A cells differentially expressed genes associated with ESC pluripotency and differentiation. A subset of differentially regulated genes is shown is Figure 4B. During differentiation, Gfp-β-cat K49A cells did not induce the expression of genes involved in mesodermal differentiation (group I), such as *cdx2* and *t-bra*. Others were neither induced in mesodermal or neuronal differentiation in Gfp-β-cat K49A cells (group II). Interestingly, many genes where upregulated selectively in mesodermal and neuronal differentiation in Gfp-β-cat K49A cells (group III). A subset of differentially regulated genes is shown in Figure 4C.

To examine in vivo differentiation potential, teratomas were produced in nude mice from the parental cell line β-Catflox/−

Figure 3. β-catMe3 and β-catAc Are Differentially Enriched at the Promoter Regions of Key Developmental Genes in ES Cells and during Differentiation

(A) qPCR analysis of *t-bra*, *sox1*, *and sox3* mRNA levels. In WT ESCs, Wnt3a reduces *sox1 and sox3* expression and induces *t-bra* expression (left). In (β-cat$^{-/-}$) ESCs, Wnt3a has no effect on the expression levels of these genes (right). Bars represent n = 3 ± SEM.

(B–D) ChIP of *t-bra*, *sox1*, and *sox3* promoters in untreated and CHIR99021-treated ESCs using mAb-K49-Ac (B), mAb-K49-Me3 (C), and mAb-β-cat-total (D). β-catMe3 and β-catAc are differentially enriched on the *t-bra*, *sox1*, and *sox3* promoters (B and C). For the total amount of β-catenin, no changes in enrichment are observed (D). After Gsk343 treatment no enrichment of β-catMe3 is detectable (C). No enrichment of β-catAc is induced (B). ChIP in anacardic acid (AA)-treated ES is used as negative control for mAb-K49-Ac (B). mAb-β-cat-total ChIP reveals that β-catenin is enriched at target gene promoters in Ezh2$^{-/-}$ cells (D). In mAb-K49-Me3 ChIP, no β-catMe3 can be detected at target gene promoters in Ezh2$^{-/-}$ ESCs (C). β-cat$^{-/-}$ ESCs are used as negative control (B–D).

(E–G) ESCs are differentiated for 4 days to mesodermal progenitors (mp d4) (E) or neuronal progenitors (np d4) (F). (E–G) ChIP in mp d4 (E), np d4 (F), and in neural stem (NS) cells (G) using mAb-K49-Ac and mAb-K49-Me3. β-catMe3, and β-catAc are differentially targeted to the lineage-specific promoters *t-bra*, *sox1*, and *sox3*. Surb7 is used as a negative control region (B–G). Bars represent n = 6 ± SEM. Statistically significant p values: *$p \leq 0.05$ and **$p \leq 0.01$ (B–G).

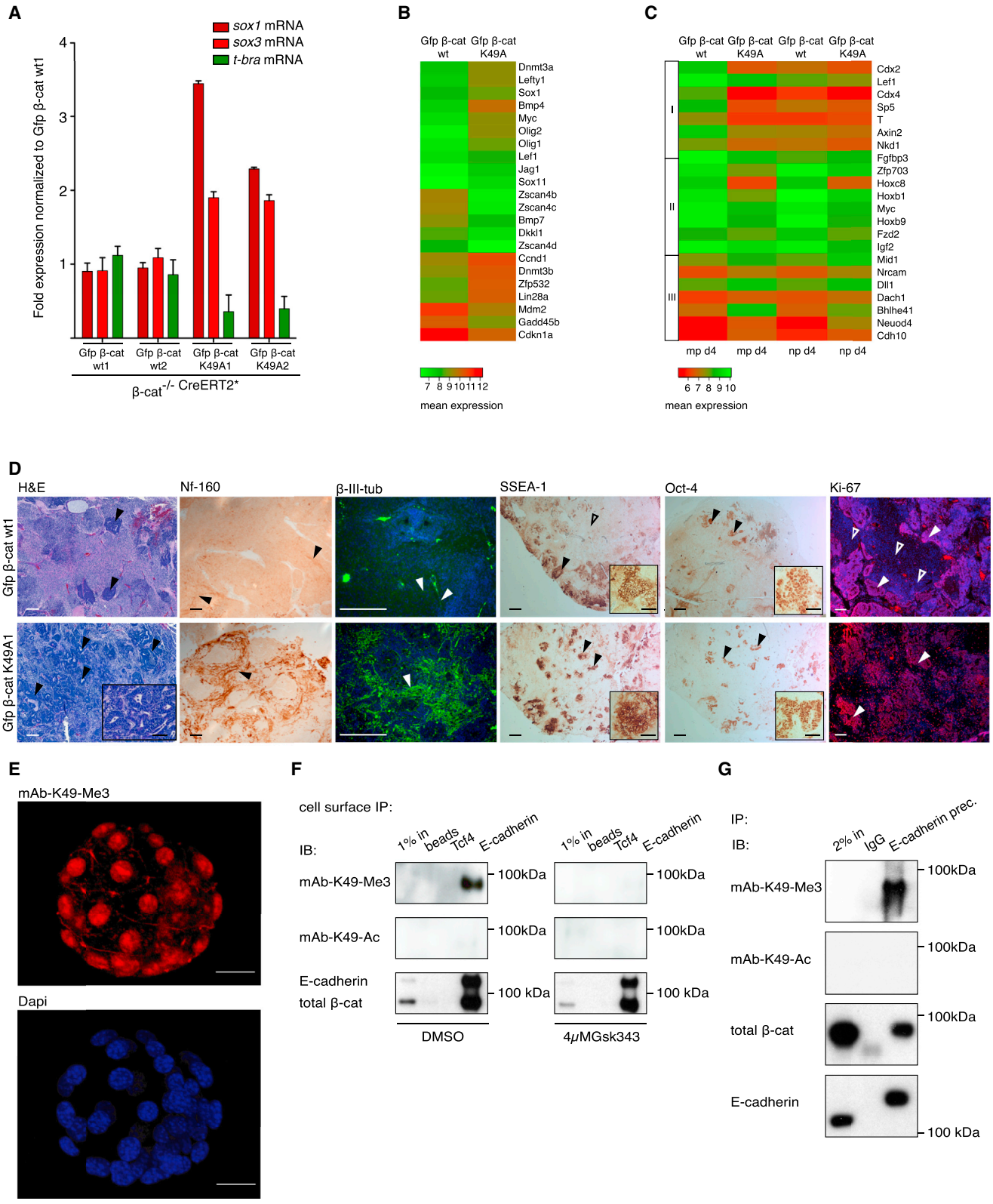

Figure 4. Lysine-49 Mutations Affect ES Cell Pluripotency and Differentiation; β-catMe3 Complexes with E-Cadherin at the Cell Membrane

(A) qPCR of *t-bra*, *sox1*, and *sox3* mRNA in β-cat$^{-/-CreERT2}$ Gfp β-cat WT and K49A ESCs. Gfp β-cat K49A ESCs show increased expression of *sox1* and *sox3* mRNA compared with Gfp β-cat WT ESCs, whereas *t-bra* mRNA levels is reduced in Gfp β-cat K49A cells.

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and from the Gfp-β-cat WT and Gfp-β-cat K49A cell lines. The parental cell line developed into benign, differentiated teratomas composed of various tissue-like structures derived from the three germ layers (not shown). In contrast, Gfp-β-cat WT and Gfp-β-cat K49A produced relatively immature teratocarcinomas that showed notable differences in the appearance of differentiated structures. Tumors derived from Gfp-β-cat WT cells contained epithelia with keratinization and mesenchymal derivatives, whereas tumors from Gfp-β-cat K49A cells exhibited predominantly neuro-rosette and neurotube-like structures with no sign of other differentiated derivatives (Figure 4D). The Gfp-β-cat K49A tumors expressed the neuronal markers Nf-160 and β-III-tubulin at much higher levels than the Gfp-β-cat WT tumors (Figure 4D). Markers for mesodermal or ectodermal cells were barely detected in Gfp-β-cat K49A tumors (not shown). However, Gfp-β-cat K49A tumors failed to downregulate the stem cell markers SSEA-1 and Oct4, and high levels of expression of both markers could be detected (Figure 4D). In addition, Gfp-β-cat K49A tumors were highly proliferative (Figure 4D).

Taken together, our in vitro and in vivo analyses demonstrate that post-translational modifications of β-catenin K49 are essential for the correct expression of key developmental genes. Mutation of K49 to alanine resulted in deregulation of t-bra, sox1, and sox3 mRNAs in ESCs, and consequently, these cells failed to differentiate into mesodermal progenitor cells and differentiated into neuronal structures.

Methylated β-Catenin Is Associated with the E-Cadherin Cell Adhesion Complex

We detected strong nuclear staining of β-catMe3 in both the trophectoderm and the inner cell mass (ICM) of mouse E3.5 blastocysts (Figure 4E). Surprisingly, β-catMe3 was detected at the plasma membrane, particularly at trophectodermal cell-cell contacts (Figure 4E). Cell surface immunoprecipitation of E-cadherin-catenin cell adhesion complexes with an antibody directed against the extracellular domain of E-cadherin revealed the presence of β-catMe3, but not β-catAc (Figure 4F). β-catMe3 was not detected in a complex with E-cadherin after treatment with the Ezh2-specific inhibitor Gsk343 (Figure 4F).

Next, we determined when β-catenin became trimethylated during its biosynthesis and intracellular transport in a complex with E-cadherin (Ozawa and Kemler, 1992). Using an antibody against the precursor polypeptide portion of E-cadherin, we showed that β-catMe3, but not β-catAc, was present in a complex with the E-cadherin precursor polypeptide (Figure 4G). These results provide convincing evidence that trimethylation of K49 is a very early event in β-catenin biosynthesis.

DISCUSSION

Wnt/β-catenin signaling is required to induce ESC mesodermal differentiation and inhibit neuronal differentiation, but how β-catenin regulates both of these differentiation pathways at the same time has been unclear. Here, we showed that β-catenin is trimethylated (β-catMe3) by Ezh2 or acetylated (β-catAc) by Cbp. We generated antibodies specific for β-catMe3 and β-catAc and showed in co-immunoprecipitation experiments that β-catMe3 is specifically present in a complex with PRC2 but not with Ash2l, a core component of the Trx group of proteins (Schuettengruber et al., 2011). These modified forms of β-catenin provide a link between Wnt/β-catenin signaling and the PRC2 chromatin-modifying complex.

β-Catenin is a substrate of PRC2 complex component Ezh2, which trimethylates β-catenin exclusively at K49 (β-catMe3). Dimethylation of β-catenin occurs at several lysine residues in armadillo repeats 4 and 5, which might regulate β-catenin stability (Lu et al., 2015). However, K49 is the only lysine acetylated by Cbp (Wolf et al., 2002), suggesting to us that K49 has a specific biological function. It is possible that Ezh2 and Cbp indirectly affect β-catenin stability by modifying components of the destruction complex.

It is well established that Wnt/β-catenin signaling inhibits the neuronal differentiation of ESCs (Haegele et al., 2003; Kielman et al., 2002; Verani et al., 2007). However, it was unclear whether β-catenin directly repressed neuronal differentiation genes. Here, we showed that β-catenin directly represses the neuronal differentiation genes *sox1* and *sox3* and that this was dependent on β-catenin trimethylation by Ezh2. Both Wnt3a and CHIR99021 treatment caused a strong β-catMe3-dependent decrease in *sox1* and *sox3* mRNA levels, at the same time as an increase in mRNA level of the mesodermal differentiation gene *t-bra*.

Our studies revealed the mechanism by which modified forms of β-catenin may counter-regulate neuronal and mesodermal differentiation gene expression. We showed that in the absence of

(B and C) Heatmap of expression levels of a subset of genes differentially regulated in β-cat$^{-/-CreERT2}$ Gfp β-cat K49A ESCs compared to β-cat$^{-/-CreERT2}$ Gfp β-cat WT ESCs (B), and after 4 days of differentiation to mp d4 or np d4 (C). Group I: mesoderm-specific genes not induced in K49A mutant mp d4. Group II: genes not induced in K49A mutant in mp d4 and np d4. Group III: genes induced in K49A mutant in mp d4 and np d4 (C). The data used to generate the heatmaps can be found in Tables S2 and S3.

(D) Immunohistochemical and immunofluorescent staining of 4-week teratomas derived from Gfp β-cat WT and Gfp β-cat K49A ESCs. H&E reveals the presence of keratinization and mesenchymal derivatives in Gfp β-cat WT tumors, whereas Gfp-β-cat K49A tumors exhibit predominantly neuro-rosette and neurotube-like structures. Nf-160 and β-III-tubulin staining indicate strong induction of neuronal differentiation in Gfp-β-cat K49A tumors. Retention of pluripotency markers in Gfp-β-cat K49A tumors is determined by measurement of SSEA-1 and Oct4 levels. Scale bars, 200 μm; insets, 50 μm. Filled arrowheads indicate positive staining. Open arrowheads indicate negative areas.

(E) Immunofluorescence with mAb-K49-Me3 reveals high nuclear levels of β-catMe3 in the trophectoderm and in the ICM of E3.5 blastocysts. In addition, β-catMe3 is detected at the cell membrane. Scale bars, 20 μm.

(F) Cell surface immunoprecipitation with E-cadherin antibody against the extracellular domain in ESCs treated with DMSO (left) or Gsk343 (right). β-catMe3, but not β-catAc, is detectable in a complex with E-cadherin (left). After Gsk343 treatment, β-catMe3 is undetectable (right).

(G) Co-immunoprecipitation with E-cadherin precursor polypeptide in ESCs reveals that β-catMe3, but not β-catAc, is present in complexes containing the E-cadherin precursor polypeptide. Immunoblot using mAb-K49-Ac and mAb-K49-Me3 against E-cadherin and β-catenin (F and G).

induction of the Wnt pathway β-catMe3, Ezh2 and H3K27Me3 were all enriched at *sox1*, *sox3*, and *t-bra* promoters. However, following activation of Wnt signaling with CHIR99021, β-catMe3, Ezh2, and H3K27Me3 were depleted from the *t-bra* promoter, whereas β-catAc and H3K27Ac were selectively enriched there. Significantly, β-catAc remained undetectable on the neuronal target gene promoters *sox1* and *sox3*, while β-catMe3, Ezh2, and H3K27Me3 were retained there, keeping the promoters in a repressed state. Based on these results, we propose that acetylation of K49 (β-catAc) is induced upon activation of the Wnt/β-catenin signaling pathway, whereas trimethylation of K49 (β-catMe3) may act upstream of Wnt signaling because β-catMe3 is detectable in the absence of ectopic Wnt induction. Thus, a β-catMe3 and polycomb-dependent repressive mechanism may act as a default pathway to maintain ESC pluripotency by repression of neuronal differentiation. Remarkably, upon induction of Wnt signaling, β-catMe3 and β-catAc binding to *sox1*, *sox3*, and *t-bra* promoters is mutually exclusive and correlates with induction of neuronal and mesodermal differentiation pathways. We showed that in the presence of Wnt signaling, β-catMe3 is replaced by β-catAc at the *sox1* and *sox3* promoters, an event that coincides with the induction of *sox1 and sox3* gene expression and the neuronal differentiation pathway. However, in mesodermal progenitor cells, β-catMe3 remains at the promoter regions of *sox1* and *sox3*, and β-catAc is enriched at the *t-bra* promoter region, resulting in β-catMe3-dependent suppression of the neuronal differentiation pathway and β-catAc-dependent activation of the mesodermal pathway. This finding clearly indicates that post-translational modification of a single amino acid residue (K49) of β-catenin directs the protein to regulate either gene repression or gene activation. Thus, the activities of methyltransferases/demethylases and acetyltransferases/deacetylases must dynamically controlled to regulate these β-catenin modifications and binding to the promoter of differentiation-specific genes.

The importance of methylation and acetylation of β-catenin K49 in regulating ESC differentiation was further demonstrated by expressing a K49A mutant in an endogenous β-catenin null background. Teratocarcinoma tumors bearing the K49A mutation exhibited a strong tendency toward the formation of neuronal structures, which we interpret, based on our results, as a loss of β-catMe3 binding to neuronal differentiation genes (e.g., *sox1*, *sox3*) that normally suppresses their expression. Alternatively, this result may be due to insufficient induction of mesoderm differentiation during tumor growth, as suggested by the absence of *t-bra* mRNA and other mesodermal markers during in vitro differentiation. It will be of interest to replace K49 with an amino acid residue that can mimic methylation.

In addition to the strong enrichment of modified forms of β-catenin in the nucleus, we also found β-catMe3 in the cytoplasm and at the cell membrane in the E-cadherin cell adhesion complex, and this interaction occurred early in the biosynthesis of the complex. Interestingly, β-catAc was barely detectable in the adhesion complex. More work is needed to characterize the function of methylated β-catenin at the cell membrane. Lysine methylation of non-histone proteins is increasingly recognized (Moore and Gozani, 2014; Zhang et al., 2012). Recently, it was reported that Ezh2 methylates the integrin-associated protein talin, which influences integrin-dependent adhesion and migration of cells (Gunawan et al., 2015).

In conclusion, our study provides a basis for understanding the molecular mechanisms through which β-catenin mediates ESC pluripotency and directs mesodermal differentiation. We describe a β-catenin function in ESCs that provides a molecular link between β-catenin and the epigenetic chromatin modifiers Ezh2 and Cbp, both of which partially exert their function on target gene chromatin through the modification of β-catenin.

EXPERIMENTAL PROCEDURES

Hybridoma Production

The peptide sequence: TAPSLSG-K-GNPEEEDVDTC-amid (Biosyntan GmbH Berlin) with the marked lysine tri-, mono-, or acetylated was coupled to maleimide-activated bovine serum albumin (BSA, for immunization), or ovalbumin (OVA, for ELISA), both purchased from Thermo Scientific. Lewis rats received six times 50 μg peptide, 100 μg CpG ODN 2006 (Imagenex), and incomplete Freund's adjuvant (GIBCO, BRL) subcutaneously (s.c.) in weekly intervals. Spleen cells and peripheral lymph nodes were fused with SP2o myeloma cells with polyethylene glycol 1500 (Roche) using standard conditions.

Chromatin Immunoprecipitation

ChIP experiments were performed as described (Hoffmeyer et al., 2012). The following antibodies were used: 5 μg of mAb-β-cat-total (produced in house), mAb-K49-Me3 (produced in house), Ezh2 (Diagenode), Suz12 (Cell Signaling), and Eed (Santa Cruz), 3 μg of mAb-K49-Ac (produced in house), and 2 μg of H3 (Abcam), H3K27Me3 (Abcam), or H3K27Ac (Abcam).

ACCESSION NUMBERS

The accession number for the micro array data reported in this paper is ArrayExpress: E-MTAB-5523.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.02.076.

AUTHOR CONTRIBUTIONS

R.K. and K.H. conceived the experiments. B.K. prepared the embryos for staining. D.J. performed immunohistochemistry on tumor sections. R.K. produced the monoclonal antibodies. K.H. performed all other experiments. R.K. and K.H analyzed the results and wrote the manuscript.

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